★ FINAL SUMMARY REPORT

ON

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BY

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"GENETIC STUDIES IN THE SPACE ENVIRONMENT" ★

NERV Studies

A repeat of the earliest successful genetic studies in the lower radiation belt was presented at " Symposium on Aerospace Radiobiology" and was subsequently published (J. Aerospace Medicine 32, 925 (1961)). Reprints are included in this report, but will be briefly summarized below.

The technique employed was a "forward-mutation" isolation technique (Woodward) previously used as a method of isolating mutant strains of Neurospora for subsequent biochemical and genetic studies. Although the technique lacks a degree of precision, it is a useful method of recovery of a large spectrum of nutritional mutants. It has been studied extensively during this grant period and will be employed as one component of a "Biosatellite Experiment" currently under development. We feel it is particularly useful for these studies since cellular phenomena related both to mutation and physiological growth can be detected and assayed.

Nerv #1 was an experiment primarily designed to expose a nuclear emulsion in order to evaluate the radiation spectrum of the lower Van Allen radiation belt. With the collaboration of NASA, small capsules containing conidia of Neurospora crassa were "hitch-hiked" and thus exposed to flight conditions for 26 minutes penetrating to an altitude of 1180 miles into the lower radiation belt.

A surprisingly high level of mutation was noted in the experimental cells when compared with three sets of control cells. A further unexpected phenomena referred to as "physiological injury" was noted in the experimental cells. This "physiological injury" phenomena could be at least partially reversed by certain organic acids which are known to function in cellular energy conversions.

It was not possible to determine what specific factor of flight, if any, contributed or caused either phenomena observed.

Bios I

In order to verify or deny the results obtained in NERV, a second flight program was developed. It was referred to as BIOS or Biological Investigation of Space and included a large number of biological experiments from our own Genetics laboratories, from Oak Ridge National Laboratory and from the General Electric Biological Laboratory as well as NASA.

In an attempt to begin dissection of the factors that might cause the effects noted in NERV, a three phase study was developed in our laboratories. The tests included 1) a repeat of the type experiment included in NERV designed to expose the complete genome as a "target" as well as allowing the detection of a "physiological death" like phenomena, 2) a back mutation experiment with multiple markers presenting a restricted target with known molecular dimensions and allowing assay of a large population in a short time. 3) A recessive lethal test system employing a balanced lethal in heterocaryon form which present the complete genome to the mutagenic agent and retaining any lethals produced for future study.

A large amount of effort was necessary to provide control "de spin" and control data. We had laboratory personnel carrying out control experiments both at the main laboratory and at the launch site. I was aboard the recovery ship, the USS Pine Island, with adequate facilities to complete preliminary studies immediately on recovery of the biological samples. As noted in an earlier report, the technical evaluation of the failure of NASA Vehicle No. 11.04 GB (E,S) and No. 11.05 GB (E,S) has already been distributed. A number of difficulties from the view of the biologists were also pointed out at that time.

Cosmic Radiation Balloon Studies

A high altitude balloon flight was made available through NASA during 1962. As discussed in a previous report, the exposure was made after much delay. Our populations were not in condition to analyze by the time we received them subsequent to the flight.

Biosatellite Project

The failure of Bios I (A and B) resulted in the eventual planning of a broad based biological study in space containing experiments from two National laboratories (Oak Ridge and Brookhaven) as well as a number of other groups including my own genetics laboratories. The possible interaction of radiation and the space environment on biological systems will be evaluated. Four basic experiments with Neurospora from these laboratories will be included: 1) Back Mutation, 2) "Woodward", 3) Recessive Lethal, and 4) Inositoless Death. These assay systems will be exposed to a dose curve ranging from 100-10,000 r as well as a shielded and unshielded control. Preliminary proceedural drafts will be included in the appendix of this report.

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Studies with Accelerator Generated Alpha Particles

Studies were carried out using the FSU Positive Particle Tandem Accelerator (Van de Graff) Capable of generating 10 MEV alpha particles. The results of these studies are formerly presented in the appendix of this report in the form of a Master's Thesis in a joint study between the Physics and Biological Sciences departments.

Control data with X-ray are presented. The data from three experiments in which cells were bombarded with a single dose of alpha particles indicate that the frequency of mutation is increased by the bombardment. Graphs are presented for both the recessive-lethal assay and inositoless death comparing the effects of x-ray and alpha particles.

Other Studies Supported (In Part) by NsG-103-61

Chemical Mutagenesis

A number of forward mutants have been isolated by means of treatment of conidia of Neurospora with such mutagens as nitrous acid, 2,6-diaminopurine, ethylmethane sulfonate and hydroxylamine. Reversion kinetics are available for certain of the stocks to be used in biosatellite experiments which provide some measure of the molecular dimensions of the genetic region presented as a mutagenic target.

"Inositoless Death" Intraallelic Recombination Analysis

Studies involving reversion studies with chemical mutagens have led to the development of a new recombination technique which should give us the capability of resolving the Neurospora chromosome to the nucleotide level. This has never before been possible with Neurospora. Such an analysis will be applied to certain of the revertants from the Biosatellite Experiment. Ultimately, a full report of this method will appear in the literature.

Isolation and Characterization of High Molecular Weight DNA from Neurospora

A number of methods of isolation of high molecular weight DNA have been developed for the first time in these laboratories (Neurospora Newsletter 3, 7 (1963)). Ultra-centrifugation analysis of such DNA indicate molecular weights up to 15,000,000. Electron microscopy studies reveal long strands of dimensions consistent with the high molecular weights obtained by means of the above mentioned analysis. One procedure employed for isolation of Neurospora DNA is indicated in the appendix of this report.

Genetic Transformation Studies in Neurospora

A number of years ago, studies were initiated in these laboratories in an attempt to demonstrate transformation in Neurospora. Techniques involved " reversion" studies similar to those to be used in the Biosatellite Experiment. No consistent results were obtained with any mutant exposed under any condition with any preparation of transforming principle employed.

Experiments in these laboratories using high molecular wieght DNA obtained from Neurospora by the above discussed procedures now appear to be successful. The technique employs the microconidiating (pe,fl) stock used in the Woodward technique but containing an aden-3 nutritional mutation. This stock is virtually free of binucleate conidia and the aden-3 stock has proved to be very stable. Using DNA isolated from wild type mycelia transformation appears to occur at the very high frequency of 4-5% of the population assayed. These studies will be pursued and reported if these results are substantiated.

Studies of Extracellular DNAse and RNAse of Neurospora crassa

Both these enzymes have been assayed in the culture media as well as associated with the growing mycelial front. The enzymes have been characterized biochemically to a limited extent. They appear to be similar in properties to enzymes isolated from the cells of other organisms.

Cytological and Cytogenetic Studies

The necessary stocks have been built to carry out back mutation studies to determine the number of copies of genetic information in a nucleus of the microconidia of Neurospora. This stock (pe:fl, aden-3) contains only one nucleus per conidium. Preliminary reversion studies have revealed no mosaic colonies representing progeny from "mutated" and "non-mutated" strands of DNA. This indicates that there is a high probability of the loss of any non-revertant DNA strand, or nucleus in any revertant clone. This work must be extended with chemical mutagens to fully clarify our kinetic data with the stocks to be employed in the Biosatellite Experiment.

Staining procedures have been developed for determination of the nuclear number of Neurospora conidia and such a procedure is included in the appendix. Data is also included on the nuclear number of conidia grown under a variety of conditions. Three stocks to be used in the Biosatellite Experiment are included, Wild Type (74A), Inositoless (498a), and a heterocaryon involving a 10:1 ratio between aden-3 and

alb, pan-2. As can be seen the age of a culture is an important consideration when it is important to keep nuclear number as low as possible.

Active Transport

We have made a preliminary observation that doses of radiation resulting in 99.99% kill of a conidial population in no way effect the rate of uptake of a labelled amino acid. This has led to a fuller characterization of the biochemical system itself prior to further radiation studies. A preliminary draft of certain of these studies are included in the appendix.

The Molecular Basis of the p-Fluorophenylalanine resistant Mutation

As mentioned above certain of the cells exposed to the experimental flight conditions of NERV #1 were unable to initiate growth on minimal media but grow normally on complete or more specifically when supplied with certain Krebs cycle intermediates. Initial studies with p-fluorophenylalanine appeared to simulate to some extent this effect of "physiological injury". This has led to extensive studies of certain mutants of Neurospora resistant to this amino acid analog. Stocks have been built by which forced heterocaryons can be prepared. Complementation does not appear to take place between alleles of such mutants although they have shown to be very closely linked. Close genetic linkage to the phen-2 locus has also been observed which has stimulated us to consider a possible operon relationship. Biochemical studies indicate that both wild type stocks and a resistant mutant (FS-188) both activate phenylalanine as well as p-fluorophenylalanine. Mutants resistant to other isomers of the analog have also been obtained. Genetic studies with these mutants are not, as yet, complete. No major differences in amino acid uptake exist between mutants and wild type conidia.

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APPENDIX

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PREPARATION OF CONIDIA FOR THE EXPERIMENT

In previous rocket or baloon experiments conidia have been harvested from agar flasks into water, filtered to remove clumps and hyphae, deposited on a membrane filter and dried. The membrane filters were stacked and placed in a special container for the experiment.

A different method of packaging conidia consists of mixing dry conidia, clumps, hyphae and all with glycine. This mixture is then packed into gelatin capsules and the capsules are placed in a suitable container for the experiment. In this method macroconidia remain dry which may be an advantage, and this method may have further advantage that more conidia can be carried in a given space.

The two methods of packaging conidia described above work equally well with macroconidial strains. However, when microconidiating strains are used, some problems arise. Microconidia are harvested with water, thus the packaging method involving the use of membrane filters is not substantially different for microconidiating strains. If the microconidia are to be bulk-packed with glycine, the procedure is different. The microconidia must be harvested with water and, therefore, must be dried before being mixed with glycine. This means that it will probably be necessary to deposit the microconidia on membrane filters, dry them, and remove them from the membranes before they can be mixed with glycine.

At this time is has not been decided if it will be better to use macroconidiating or microconidiating strains. Also, the manner in which the conidia will be packaged during the experiment has not been finally determined.

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RECESSIVE LETHAL TECHNIQUE

Introduction

This technique is used to determine the frequency of recessive lethal mutations in Neurospora crassa. This procedure makes use of the fact that nuclei carrying recessive lethal mutations may be maintained and studied if they are incorporated into a heterocaryon. The frequency and possibly the nature of the recessive lethal mutations may be used as a measure of the extent of damage to the genome.

General Description of the Method

Conidia from a strain which is a balanced heterocaryon are used in this technique. A balanced heterocaryon contains nuclei of two auxotrophic strains which complement each other to the extent that the heterocaryon is able to grow on minimal medium. In addition each nuclear type must have markers which enable the two types to be visually distinguished from each other and from wild type when they occur as homocaryons on the same plate.

Conidia from the balanced heterocaryon are exposed to the experimental conditions and plated on minimal medium. Only the heterocaryotic conidia which survive the treatment will be able to form colonies on minimal medium. These colonies are isolated from each other and allowed to grow and conidiate. The conidia from these isolates are then plated on a medium on which the homocaryotic as well as the heterocaryotic conidia are able to grow. Those isolates which fail to produce one type of colony having a homocaryotic origin are scored as recessive lethals. The frequency of recessive lethals in the isolated colonies will be used as a measure of the extent of mutagenic action by the treatment. Further screening of the recessive lethals may be carried out to determine the proportion which have relatively simple nutritional requirements.

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The proportion of nuclear types in Neurospora heterocaryons as determined by plating.
Genetics 38, 518 (1953)

The Recessive Lethal Test in the Biosatellite Program

1. Stocks. The stock used in this experiment will be a heterocaryon prepared from an adenine (ad-3) strain and an albino pantothenic strain (al-2,pan). This heterocaryon will be prepared according to the method of Pittenger and Atwood (1955) and should have a nuclear ratio of about 1:1. Heterocaryons of other stocks may be used if required.

2. Growth of stocks. For the experiment the heterocaryon will be grown in 125 ml. Erlenmeyer flasks containing 40 ml. of the following medium:

20 grams agar
20 grams sucrose
1000 ml. 1X Vogel's solution

Each flask is inoculated with one drop of a heavy conidial suspension or with a plug of mycleium from the heterocaryon. The stocks from which the heterocaryon is prepared are maintained on minimal media supplemented with the required growth factor.

3. Harvesting of conidia. In contrast to the other techniques used in the Biosatellite program this test uses only macroconidial strains. For the experiment conidia are harvested from the growth flasks by means of a sterile inoculating n eedle.

4. Preparation of conidia from the experiment. At the present time it has not yet been determined how the conidia will be packaged during the experiment. Problems related to this are discussed in a separate paper entitled "Preparation of Conidia for the Experiment".

5. Post exposure treatment of conidia. After the conidia have been exposed to the experimental or control coniditions they are suspended in water. The suspension of conidia may be filtered through a glass wool or cotton filter if it is necessary to remove conidial clumps or hyphae. A conidial count is made. The conidia are diluted and plated by spreading on plates containing the following minimal medium:

20 grams sorbose
2 grams sucrose
20 grams agar
1000 ml. 1X Vogel's solution

After the number of colonies are counted at least 200 from each treatment or control are transferred to slants containing minimal medium. These isolated colonies are incubated at 25°C until all have formed conidia. Conidia from each of these isolates are serially spread on six plates containing plating medium which allows the growth of each homocaryotic type. In the case of the ad-3/al-2,pan heterocaryon, the following medium would be used:

20 grams sorbose
20 grams agar
1 gram fructose
1 gram glucose
5 mg adenine sulphate
50 mg calcium pantothenate
1000 ml. 1X Vogel's solution

These plates are incubated at 25°C for 7 to 10 days. The plates spread from each isolate are examined to determine if they contain colonies of both possible homocaryotic

types. Those isolates which contain both types are scored as nonlethals, while those isolates which lack homocaryotic colonies for one type are scored as recessive lethals. The isolates containing recessive lethals will be further tested on plates to determine if the lethal bearing nuclei have nutritional requirements which can be satisfied by some substance or combination of substances.

5. Interpretation of data. From the conidial and plate counts described above it should be possible to determine the survival and recessive lethal frequencies. The interpretation which will be placed on the results of attempts to determine a metabolite requirement in the recessive lethals will depend on the results of such screening procedures.

Equipment

Equipment lists for the recessive lethal test have not yet been prepared.

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THE FILTRATION CONCENTRATION ('Woodward') TECHNIQUE

Introduction

The filtration concentration technique is a procedure for the recovery of spontaneous or induced auxotrophic mutations occurring in conidia of Neurospora. The filtration concentration technique makes use of the fact that germinating conidia may be selectively filtered from a suspension of conidia. By means of repeated filtrations of a suspension of conidia in minimal medium, the ratio of auxotrophic to prototrophic conidia in the filtrate will be increased.

General Description of the Method

Conidia are harvested and subjected to the mutagenic treatment being used in the experiment. Following the mutagenic treatment the conidia are inoculated into a flask containing liquid minimal medium. The conidia are incubated with agitation and aeration. When germination is visible the suspension is passed through a filter designed to retain germinated conidia and pass ungerminated conidia. When new germination is visible the filtration process is repeated. After these filtrations have been repeated a number of times the interval between visible germination will become extended to several days or more. At this time it may be assumed that most of the prototrophic conidia have germinated and been filtered from the suspension. At this time the suspension is ready to be assayed for the number and type of surviving conidia. This assay is usually made by plating the suspension on complete medium in order to obtain a high germination of the viable conidia remaining in the suspension. If the plates indicate that there may be auxotrophic mutants present, then the colonies on the plates must be isolated and tested for their phenotypes.

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Selection of particular biochemical mutants in Neurospora from differentially germinated conidial filtrates.
Genetics 37, 637 (1952)

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The separation and isolation of particular biochemical mutants of Neurospora by differential germination of conidia, followed by filtration and selective plating.
Proc. Nat'l. Acad. Sci., 40, 191 (1954)

The Filtration Concentration Technique as Applied to the Biosatellite Program

1. Stocks. One of the following strains of Neurospora crassa will be used: pe,fl or 74A. It has not yet been determined if a microconidiating or a macroconidiating strain will be most suitable in this technique.

2. Growth of stocks. If the pe,fl strain is used it will be grown in 125 ml. flasks containing 40 ml. of the following medium:

20 grams agar
20 grams sucrose
1 gram yeast extract
1 gram malt extract
0.5 grams liver extract
0.1 gram casa amino acids
0.1 gram bacto-peptone
1000 ml. 1X Vogel's solution

Each flask is inoculated with one drop of a conidial suspension and incubated at 20° C for 14 days.

If the 74A strain is used, it will be grown in 125 ml. flasks containing 40 ml. of the following medium:

20 grams agar
20 grams sucrose
1000 ml. 1X Vogel's solution

Each flask is inoculated with one drop of a conidial suspension and incubated for five days at 25° C.

3. Harvesting of conidia. The procedure used for harvesting conidia will depend on whether a microconidiating or a macroconidiating strain is used and on how the conidia will be packaged for the experiment. The problems involved in these differences have been discussed in a separate paper.

4. Preparation of conidia for the experiment. There are two methods of packaging conidia being considered. The possible advantages and disadvantages of these methods are discussed in the separate paper mentioned above.

5. Treatment of conidia following exposure. Following exposure of the conidia to the experimental or control conditions the conidia are suspended in water, filtered if necessary to remove clumps or hyphae, and a conidial count is made. A predetermined number of conidia are inoculated into a one liter Erlenmeyer flask containing 400 ml. of the following medium:

20 grams sucrose
1000 ml. 1X Vogel's solution
an antibiotic (type and amount to be determined)

The flask is closed with a cotton plug and the upper 1/3 of the flask covered with aluminum foil. The flask is placed on a shaker where the culture will remain except when it is being filtered.

6. Filtration. During the first two or three days of incubation the culture must be filtered at intervals of 12 hours. After the third day when the amount of germination is considerably reduced the interval between filtrations may be increased to 24 hours. These time intervals are based on work done with the pe, fl strain. The intervals between filtrations will require some modification if conidia of 74A are used. The filtration is made by use of filter flasks. Filter flasks consist of one liter Erlenmeyer flasks which have a layer of cloth placed over the mouth of the flask, the cloth forced inward until it assumes a cup shape, and secured to the neck of the flask with a rubber band. The actual cloth to be used in the filters has not been determined. Woodward et. al. used two layers of cheese cloth. In this laboratory a commercial grade of nylon organdy and a monofilament nylon mesh (Nitex) have been used. A critical

test of the relative efficiency of the different filter cloths has not yet been made.

The flasks are fitted with cotton stoppers and the upper 1/3 of the flask is covered with aluminum foil. All flasks used in this experiment are autoclaved at 18 lbs. pressure for 25 minutes.

Prior to the filtration the culture flasks are removed from the shaker and placed in a sterile transfer room where the exterior of the flask is exposed to germicidal UV light for thirty minutes. The filtration consists of pouring the contents of the culture flask through the filter into the filter flask. The filter is removed from the filter flask, the cotton plug placed in the neck and the aluminum foil replaced. The new flask is placed on the shaker for another period of incubation.

7. Termination. After the culture of conidia have been grown for the required number of days they are harvested by filtering the contents of the culture flask on a membrane filter. The conidia on this filter are resuspended by shaking the filter in 10 ml. of water.

8. Plating. One ml. aliquots of the suspension are added to 100 ml. of melted agar sorbose-fructose-glucose minimal and complete media and these mixtures are poured into petriplates. These plates are incubated at 25° C.

9. Screening. In any experiments where mutations are produced there should be a higher colony count on the complete plates than there is on the minimal plates. The greater the difference between the counts the greater has been the genetic damage to the conidia and/or the greater has been the resolving power of the filtration concentration procedure. To determine the frequency of mutants in the colonies on the complete plates the colonies must be transferred to slants of complete medium. After these isolates have grown they are screened on minimal medium to determine the proportion of wild type in the isolates. Those isolates which fail to grow as a wild type may be further screened on different classes of compounds in an attempt to determine the exact nature of the nutritional requirement.

10. Relation of mutation number to mutation frequency. In some way the number and frequency of auxotrophic mutants recovered from each sample subjected to the filtration procedure must be related to the extent of mutation produced by the experimental conditions. At the present stage of work on this project the manner in which this

relationship will be determined has not been worked out.

Equipment

Equipment list for the filtration concentration technique have not yet been prepared.

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BACK MUTATION TECHNIQUE

Introduction

This procedure is used to assay the frequency of spontaneous or induced reversion to prototrophy by a given auxotrophic mutant. This frequency may be used as an index of the total radiation received by population of conidia.

General Description of the Method

The required number of macroconidia or microconidia are harvested from growth flasks into water, buffer, or liquid growth medium. These conidia may be dried or kept as a suspension during the mutagenic treatment as the experimental circumstances require. Prior to experimental treatment, the sample of conidia is divided so that experimental and control samples will be available. Following the mutagenic exposure the experimental and control samples of conidia are assayed to determine the number of viable conidia and the frequency of back-mutation. Viability is determined by diluting and plating the conidia on a complete medium. Reversions are assayed by plating the conidia on minimal medium.

The chief advantage of the back-mutation technique lies in the relative ease with which the reversions may be identified and isolated from the non-reverted cells. The principal disadvantage of the back-mutation technique is the fact that the number and type of genetic changes which can produce a reversion may be limited and highly specific and thus the reversion frequency may not be a good index of the total amount of damage incurred by the biological system.

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Cold Spring Harbor Symp. Quant. Biology XI, 215 (1946)

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Reverse mutation and adaptation in leucineless Neurospora.
Proc. Nat'l. Acad. Sci., 32, 163 (1946)

The Back Mutation Test in the Biosatellite Program

1. **Stocks.** The stock used in the back-mutation test will be either FS-199 (col, ad-3, inos) or FSR-5 (pe,fl, ad-3, inos). These strains have been selected because they are known to give relatively high reversion rates with X-ray .

2. **Growth of Stocks.** If the strain FSR-5 is used it will be grown in 125 ml. flasks containing 40 ml. of the following medium:

20 grams agar
20 grams sucrose
1 gram yeast extract
1 gram malt extract
0.5 gram liver extract
0.1 gram casa amino acids
0.1 gram bacto-peptone
0.1 gram inositol
0.1gram adenine sulfate
1000 ml. 1X Vogel's solution

Each flask is inoculated with one drop of a conidial suspension and incubated at 20°C for 14 days.

If strain FS-199 is used, it will be growth in 125 ml. flasks containing 40 ml. of the following medium:

20 grams agar
20 grams sucrose
5 grams yeast extract
5 grams malt extract
0.3 grams casein
1 gram choline chloride
0.1 gram inositol
0.1 gram adenine sulfate
1000 ml. 1X Vogel's solution

Each flask is inoculated with one drop of a conidial suspension and incubated at 25° C for five days.

3. Harvesting of conidia. The procedure for harvesting conidia will depend on whether a microconidiating or macroconidiating strain is used and on how the conidia will be packaged for the experiment. The problems involved in these differences have been discussed in a separate paper.

4. Preparation of conidia for the experiment. There are two ways that the conidia may be carried during the experiment. The problems involved using these methods have been discussed in the separate paper mentioned above.

5. Post exposure treatment of conidia. Following exposure of the conidia to the experimental or control conditions the conidia are resuspended in water, filtered if necessary to remove clumps or hyphae, and a cell count is made. On the basis of the cell count an aliquot of the suspension is diluted and platings of the various dilutions are made in complete medium to determine the viable population of the suspension. Undiluted aliquots of the conidial suspension are plated on media which will allow the growth of revertants of the ad-3 or inos genes.

6. Interpretation of data. With the information obtained from plate counts, it should be possible to determine the proportion of conidia killed and the frequency of reversion at each locus being studied.

Equipment

Equipment lists for the back-mutation tests have not yet been prepared.

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MUTATION RECOVERY THROUGH "INOSITOL-LESS DEATH"

Introduction

The "inositol-less death" technique is a method for the selection of induced and spontaneous auxotrophic mutants. This technique is based on the fact that conidia of an inositol-requiring strain die rapidly when they germinate in media not containing inositol. However, when the conidia of the inositol-requiring strain contain a second auxotrophic mutation "inositol-less death" is greatly reduced. Apparently, the second mutation stops germination before "inositol-less death" occurs. Using this technique it should be possible to selectively recover spontaneous or induced auxotrophic mutants.

General Description of the Method

The required number of macroconidia or microconidia of the inositol-requiring strain are harvested into water, buffer, or liquid growth medium. These conidia may be dried or kept as a suspension during the mutagenic treatment as the experimental circumstances require. Following the mutagenic treatment, the conidia are incubated for a few hours in a liquid minimal medium supplemented with a small amount of inositol (0.25µg/ml). This step permits the conidia to start germination and allows for the phenotypic expression of secondary auxotrophic mutations. After this incubation period, the germinated conidia are removed by filtration. The remaining conidia are washed with water to remove the inositol and incubated in minimal medium to permit " inositol-less death" to take place in those conidia capable of germination. After two to six days' incubation the medium is enriched with concentrated complete medium. The colonies growing at this time are isolated and tested for their phenotype.

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Efficient method for selection of auxotrophic mutants of Neurospora.
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Cell death and "unbalanced growth" in Neurospora.
J. General Microbiol., 18, 658 (1958)

Inositol-less Death as Applied to the Biosatellite Program

1. Stocks. One of the following strains of Neurospora will be used:

pe, fl, inos or col, inos.

2. Growth of stocks. If the pe, fl, inos strain is used it will be grown in 125 ml. flasks containing 40 ml. of the following medium:

20 grams agar
20 grams sucrose
1 gram yeast extract
1 gram malt extract
0.5 grams liver extract
0.1 gram casamino acids
0.1 gram bacto-peptone
0.1 gram inositol
1000 ml. 1X Vogel's solution

Each flask is inoculated with one drop of a conidial suspension and incubated at 20° C for 14 days.

If the col, inos strain is used, it will be grown in 125 ml. flasks containing 40 ml. of the following medium:

5 grams yeast extract
5 grams malt extract
0.3 grams casein
1.0 grams choline chloride
20 grams sucrose
20 grams agar
0.1 grams inositol
1000 ml. 1X Vogel's solution

Each flask is inoculated with one drop of a conidial suspension and incubated for five days at 25°C.

3. Harvesting of conidia. The procedures for the harvesting of the conidia will depend on the manner in which the conidia are to be packaged during the experiment and on whether the strain used is microconidiating or macroconidiating. These problems have been discussed elsewhere under the title of Preparation of Conidia for the Experiment.

4. Preparation of conidia for experiment. Details of this procedure will not be given here for the same reasons given under harvesting of conidia. Detailed procedures will be worked out later.

5. Treatment of conidia following exposure. The number of experimental and control samples has not yet been worked out, however, they will all receive the same treatment after the exposure. Following the exposure of the conidia to the experimental or control condiations, a portion of the conidia will be suspended in minimal medium with inositol, filtered (necessary with the glycine-conidia mixture but not with the membrane filter-carried conidia), and counted. After aliquots of the suspension are removed for plating to determine the viable population, the conidial suspension is incubated with shaking.

6. Filtration and washing. At the end of the six hours of incubation, the suspension will be filtered through a cotton, glass wool, or nitex filter. The filtrate will have some powdered agar added to it and it will be washed three times with water by means of centrifugation in a clinical centrifuge. Following washing the conidia are re-suspended and counted and the volume of the suspension is adjusted to give a conidial count of 10^7/ml.

7. Plating and incubation. 1 ml. aliquots of the suspension are added to 100 ml. of melted agar sorbose-fructose-glucose minimal medium and this mixture is poured into petri plates. These plates are incubated at 30°C.

8. Overlayering. At the end of 4-6 days, the plates are overlayered with a 2X inositol supplemented complete medium. These plates are incubated at 30°C.

9. Isolation of colonies. After four days of incubation the colonies which have appeared on the plates are transferred to complete slants. These isolates will be screened on complete and inositol supplemented minimal medium to determine the proportion which have a secondary mutation.

10. Relate mutation number to frequency. In some way the number and frequency of secondary mutations must be related to the population surviving the experimental conditions. At the present time the manner in which the mutation frequency in the survivors will be determined has not been worked out.

Equipment

Equipment lists for these "inositol-less death" techniques have not yet been prepared.

Genetics Laboratories

12 December 1963

ARG

PROCEDURE FOR STAINING NUCLEI OF NEUROSPORA CRASSA

1. Clean slides thoroughly in 70% alcohol. Dry completely.
2. Place a small drop of liquid albumin on slide, spread with finger to cover smear area with thin coat, allow to air dry (should be covered loosely during drying to prevent dust, etc. from the air adhering to the slide.)
3. Preparation of sample to be stained:
   a) Conidia from culture grown on solid media: With a sterile inoculating needle, take mycelia from the culture and made a fairly heavy suspension in Carnoy's fixative. Filter through a spun glass conidial filter. Smear duplicate slides with the filtered suspension. Allow to dry and proceed with Step 4.
   b) Conidia or mycelia from a liquid suspension: Smear duplicate slides with 1-2 drops of a fairly heavy suspension. Allow to dry. (Drying may be speeded somewhat by aiming a stream of cool air at the slide. Do not use heat). Proceed with Step 4.
4. Place slides in Carnoy's fixative for 20 minutes.
5. Remove from Carnoy's and place directly into 1N HCl at 60° C for 15 minutes.
6. Remove from 1N HCl and wash twice in distilled H_2O baths at 60° C.
7. Place in chrom-alum-haemotoxylen stain at 60° C for 15 minutes.
8. Remove from stain and rinse thoroughly in distilled H_2O.
9. Place in 45% acetic acid for 15 minutes.
10. Remove from acetic acid. With a pipette and clean 45% acetic acid, rinse slide.
11. Place slides on paper towels. Put one drop 45% acetic acid on smear, place clean cover slip on, and blot gently with tissue.
12. Seal edges of cover glass completely with nail polish. Allow to dry.
13. Keep refrigerated in covered petri dishes until ready to observe microscopically. (Should be observed within 24 hours for best results).

NOTES:

1. **Carnoy's Fixative (Should be made fresh daily).**

   a. 100% ethanol 60 ml.
   b. Chloroform 30 ml.
   c. Glacial acetic acid 10 ml.

2. **Chrom-alum haematoxylin Stain (Should be made fresh every 3-4 days).**

   a. Dissolve 1.5 grams chrom-alum (chromium potassium sulfate) in 50 ml. distilled H_2O.

   b. Dissolve 0.5 grams haematoxylin in 50ml. distilled water.

   c. Combine a and b.

   d. Add 2 ml. of 5% potassium dichromate and 2 ml. of 5% H_2SO_4.

   e. Ripen 2 days in light (in corked bottle or flask).

3. It has been found that conidia and smeared slides may be kept refrigerated in Carnoy's fixative for 24-48 hours with no deleterious effects. This can be of value when you are taking samples in series and want to stain all at the same time, or if you do not have time to stain at the time you take the sample.

Genetics Laboratories
Florida State University

ARG

Neurospora crassa Mutant	Days Incubated	Incubation Temperature	Media	Total Conidia Counted	Total Nuclei Counted	Average No. Nuclei/Conidia	% Conidia with No Nuclei	% Conidia with One Nucleus	% Conidia with Two Nuclei	% Conidia with Three Nuclei	% Conidia with Four Nuclei	% Conidia with Five Nuclei	% Conidia with Six Nuclei
Wild Type													
(74 A)	2	25°	Min	182	496	2.72	0	21.4	27.5	19.8	18.7	7.7	4.4
(74 A)	4	25°	Min	163	556	3.41	0	9.8	25.8	19.0	22.0	13.5	5.5
(74 A)	7	25°	Min	182	508	2.78	0	15.4	35.0	23.5	13.7	8.8	1.65
(74 A)	10	25°	Min	176	443	2.51	1.71	23.9	31.8	23.3	10.8	7.4	0
(74 A)	2	30°	Min	173	521	3.01	0	12.1	30.6	20.2	26.0	6.35	2.9
(74 A)	4	30°	Min	213	663	3.11	0	10.3	33.0	19.2	16.9	12.2	7.05
(74 A)	7	30°	Min	204	540	2.65	2.45	9.3	40.8	25.5	14.7	5.4	1.97
(74 A)	10	30°	Min	173	425	2.38	4.65	19.7	26.6	28.3	9.25	10.4	5.79
(74 A)	2	35°	Min	179	522	2.91	0	12.8	34.0	23.5	17.3	6.7	3.9
(74 A)	4	35°	Min	244	507	2.08	0	17.1	68.5	11.2	3.34	.83	0
(74 A)	7	35°	Min	260	555	2.14	0	18.0	57.2	18.4	5.78	0	.38
(74 A)	10	35°	Min	245	509	2.07	4.1	13.5	57.6	20.8	3.6	4.1	0
(74 A)	2	25°	Com	293	536	1.89	0	44.7	34.8	15.0	4.4	.7	0
(74 A)	4	25°	Com	312	525	1.68	.64	47.6	37.8	11.5	2.3	.3	0
(74 A)	7	25°	Com	335	595	1.79	1.79	41.5	37.9	12.2	4.8	1.4	.2
(74 A)	10	25°	Com	381	620	1.63	1.1	48.8	39.4	8.2	2.4	.3	0
(74 A)	2	30°	Com	201	496	2.46	0	17.9	47.8	15.4	12.0	5.5	0
(74 A)	4	30°	Com	261	596	2.28	0	18.0	46.4	17.6	12.2	3.1	1.2

% Conidia with Seven Nuclei	% Conidia with Eight Nuclei	% Conidia with Nine Nuclei	% Conidia with Ten Nuclei	Approx. % Diffuse Nuclei
0	0	0	0	75
1.8	1.8	0	.6	50
.55	0	1.1	0	25
1.14	0	0	0	<5
.58	.58	.58	0	75
0	0	.47	0	50
0	0	0	0	20
0	5.79	0	0	<10
0.	1.12	.56	0	85
.42	.42	0	0	25
0	0	0	0	<10
0	0	0	0	<10
.4	0	0	0	75
0	0	0	0	50
0	0	0	0	15
0	0	0	0	<10
.1	.5	0	0	80
1.2	.4	0	0	50

Neurospora crassa Mutant	Days Incubated	Incubation Temperature	Media	Total Conidia Counted	Total Nuclei Counted	Average No. Nuclei/Conidia	% Conidia with No Nuclei	% Conidia with One Nucleus	% Conidia with Two Nuclei	% Conidia with Three Nuclei	% Conidia with Four Nuclei	% Conidia with Five Nuclei	% Conidia with Six Nuclei
Inositoless													
(498 a)	2	25°	****	255	563	2.20	0	21.9	48.1	20.0	7.4	1.5	.7
(498 a)	4	25°	****	233	539	2.31	0	18.4	47.6	23.2	9.9	.9	0
(498 a)	7	25°	****	239	539	2.28	.4	19.6	45.1	25.5	7.5	1.2	.4
(498 a)	10	25°	****	222	474	2.13	.4	23.4	47.7	20.7	5.4	.4	0
(498 a)	2	30°	****	254	507	1.99	0	28.7	48.0	18.4	4.3	.4	0
(498 a)	4	30°	****	257	499	1.94	0	34.6	43.9	15.6	5.4	.4	0
(498 a)	7	30°	****	254	498	1.92	0	31.8	56.3	13.4	3.5	.9	.4
(498 a)	10	30°	****	270	519	1.92	.4	33.3	45.6	15.6	4.9	.4	0
(498 a)	2	35°	****	301	563	1.87	0	37.2	43.5	16.8	2.7	.7	0
(498 a)	4	35°	****	293	538	1.83	0	40.9	38.9	16.7	3.4	.3	0
(498 a)	7	35°	****	290	562	1.93	0	35.5	41.8	16.2	6.5	0	0
(498 a)	10	35°	****	305	565	1.85	0	38.0	38.7	17.4	5.2	.7	0
Heterocaryon													
10:1	2	25°	Min	255	576	2.26	0	24.0	39.6	24.7	10.6	.8	.4
10:1	4	25°	Min	238	476	2.0	0	22.8	58.6	16.0	1.9	0	0
10:1	7	25°	Min	241	495	2.05	0	27.0	51.1	16.6	5.0	1.3	.5
10:1	10	25°	Min	194	522	2.68	0	9.3	34.6	36.1	15.5	5.1	0
10:1	2	30°	Min	236	554	2.35	0	21.2	41.2	27.1	6.4	2.6	.9
10:1	4	30°	Min	218	522	2.39	0	18.0	43.2	26.2	9.7	1.8	.5

% Conidia with Seven Nuclei	% Conidia with Eight Nuclei	% Conidia with Nine Nuclei	% Conidia with Ten Nuclei	Approx. % Diffuse Nuclei
0	0	0	0	85
0	0	0	0	60
0	0	0	0	20
.4	0	0	0	<10
0	0	0	0	90
0	0	0	0	50
0	0	0	0	20
0	0	0	0	<5
0	0	0	0	85
0	0	0	0	30
0	0	0	0	10
0	0	0	0	<5
0	0	0	0	90
0	0	0	0	60
0	0	0	0	30
0	0	0	0	<10
.5	0	.5	0	90

PROCEDURE FOR ISOLATION OF HIGH MOLECULAR WEIGHT DNA FROM NEUROSPORA

July 1963

Mycelial Powder:

Cultures of Neurospora grown for 72 hours in liquid media under aeration are poured through several layers of bleached cheesecloth, the mycelia squeezed dry. Small bits are dropped into liquid nitrogen and ground to a fine powder in an automatic mortar and pestle. If the powder is not to be used immediately, it is stored in a deep freeze at -20° C.

Isolation of Na-DNA (Cold):

100 grams of frozen mycelial powder are suspended in 25 ml. of cold 0.1M NaCl and 4 volumes of ethanol/ether are added (1/1). The solution is shaken vigorously for 15 minutes and then centrifuged at 3,000 rpm. The supernatant is discarded and the residue re-extracted in the same manner.

The final pellet is suspended in 50 ml. of tris buffer (pH 8.0; 0.2 M) and to this is added an equal volume of 10% Aerosol O.T. (Fisher Scientific Company) and the resulting suspension is stirred overnight in the cold. The nucleic acids are extracted by adding NaCl to 1M, stirring for 1/2 hour , and centrifuging at 3,000 rpm for 15 minutes. To the supernatant is added 2 volumes of 95% ethanol (-20° C). DNA may be wound out on a clean glass rod or centrifuged and the pellet stored in the deep freeze until needed.

For quantitative extraction of DNA the NaCl precipitate may be extracted with 1M NaCl centrifuged and ethanol added to the supernatant. Three or four extractions usually remove all the DNA.

Deproteinization (After Marco and Butler, J. Biol. Chem., 190, 165 (1951)):

The DNA (as an alcoholic precipitate) is collected by centrifugation (3,000 rpm ; 15 minutes), washed twice with 70% ethanol and dissolved in as small a volume as possible of 0.1M NaCl. The NaCl concentration is brought to 1M and 0.2 volumes of sodium dodecyl sulfate (5 grams in 100 ml. of 45% ethanol) is added with gentle stirring. The solution is allowed to stand 1 hour at room temperature and then two days in the cold. The solution is centrifuged and the supernatant precipitated by the addition of 2 volumes of cold 95% ethanol as before.

Removal of RNA and Single Stranded DNA (After Aldridge, Nature 195, 284 (1962)): The DNA is collected, washed with 70% ethanol and dissolved in 0.1M NaCl. The NaCl concentration is brought to 2 M and the pH adjusted to 5.0 with 0.2M acetate buffer and 0.25 volumes of 0.05 indium trichloride (freshly made) is added. After standing at room temperature for 30 minutes, the mixture is centrifuged and the pellet discarded. The supernatant is dialyzed against 1M NaCl for 24 hours in the cold and the pH adjusted to 13.0 with NaOH and the solution incubated at 40°C for 12 hours. The solution is dialyzed against 1M NaCl and precipitated with ethanol.

Molecular Weight Determination:

The sample is dissolved in NaCl (0.15M), NaCitrate (0.015M), pH 7.0, at a concentration of 100γ/ml. Sedimentation studies are carried out in a Spinco Model E analytical ultracentrifuge. (Rotor AF, cell with Kel-F centerpiece) at 35,000 rpm. (The curve is no longer linear above 40,000 rpm; Stuy, J.H., personal communication). Records of the sedimentation rate are made by photography.

Sedimentation velocity is calculated from the equation:

$$S_{20} = \left(\frac{dx}{x}\right) / w^2\,dt \qquad S_{20} = S \text{ at } 20^\circ\text{ C.}$$

$$S_{20} = \frac{\ln x_2 - \ln x_1}{w^2 (t_2 - t_1)}$$

$$\text{where } w^2 = (4)\ (3.1416)^2\ (\text{RPM})^2 / \text{sec}^2$$

The photographic record is analyzed on a recording Spinco analytical densitometer and the S_{20} ($X\ 10^{-13}$) is calculated directly from the chart readings.

Sample calculations:

12' = 1.58

20' = 1.98

36' = 2.82

Magnification factor $1.60 / 14.65 = 0.10922$

$$S_{20} = \frac{\log \left[(40.6)\quad(0.10922) + 5.71\right] - \log \left[(0.950)\ (0.10922) + 5.71\right]}{2.1717 \times 10^{10}}$$

$S_{20} = 11.33 \times 10^{-3}$ = approximately 1,000,000 molecular weight

Base Ratio Analysis (After Anderson and Ladd, Biochim. Biophys. Acta., 55, 275 (1962)):

A one mg. sample is placed in a thick glass ignition tube. Two ml. of formic acid is added and nitrogen is bubbled through the solution for 10 minutes. The tube is sealed with an oxygen torch and heated at 175° C for 2 hours. After cooling, the tube is opened, placed inadessicator over KOH and evaporated. The white residue is taken up in 0.1 N NCl, diluted to pH 7.0 and absorbed onto a column of Dowex 1X 10 acetate (150 cm. x 3.14 cm^2).

The bases are eluted with sodium acetate, acetic acid buffer (pH 4.4; 0.2M to 2M graded linearly), and 10 ml. fractions are collected. The eluate is continuously monitored by a recording absorption meter at 265 mμ. The fractions containing each individual base are pooled, brought to a standard volume and the concentration determined against standard concentrations in a Beckman DU spectrophotometer.